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Greater hemocyte bactericidal activity in oysters (*Crassostrea virginica*) from a relatively contaminated site in Pensacola Bay, Florida

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Abstract

Bivalve mollusks such as *Crassostrea virginica* inhabiting polluted estuaries and coastal areas may bioaccumulate high concentrations of contaminants without apparent ill effects. However, changes in putative internal defense activities have been associated with contaminant accumulation in both experimental and long-term field exposures. In an effort to elucidate these relationships, 40 oysters were collected from Bayou Chico (BC) and East Bay (EB) in Pensacola Bay, FL, two estuaries known to differ in the type and magnitude of chemical contaminants present. Oyster tissue concentrations of metals, tri- and dibutyltin (TBT, DBT), polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) were measured in individual oysters, as were hemocyte counts (HCs), hemocyte bacterial killing indices (KI), serum lysozyme (LYS) and serum protein (PRO) levels. Average HC, KI, LYS and PRO were significantly higher in BC oysters, which also had significantly higher tissue concentrations of total trace metals, butyltins (BTs), PAHs, PCBs, pesticides, and Mn, Cu, Zn and Sn. EB oysters had low organic contaminant levels and no detectable BTs, but significantly higher concentrations of Al, Cr, Fe, Ag, Cd, and Hg. Simple correlation analysis between specific defense measurements and specific chemical analytes showed specific positive relationships that corroborated previous findings in other FL estuaries. Canonical correlation analysis was used to examine relationships between defense measurements and tissue metals using linearly combined sets of variables. Results were also consistent with previous findings—the highest possible canonical correlation was positive: $r = 0.864$, $P < 0.0019$ among canonical variables composed of HC, KI and LYS for defense, and Fe, Cu, Ag, Cd, Sb, Sn, Ni, Pb and Hg for metals.
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1. Introduction

Exposure of marine organisms to anthropogenic chemicals can alter vital physiological processes, such as hemocyte activities, needed to mount an effective defense against pathogens (Cheng, 1977; Feng, 1988) and to perform other physiological functions (Feng et al., 1977; Morton, 1983; Watanabe, 1983). Populations of eastern oysters, *Crassostrea virginica*, on the eastern US coast and the Gulf of Mexico have declined, partly due to pathogens such as *Perkinsus marinus* (Soniat, 1996; Andrews, 1996). Oyster declines have been concomitant with increased industrial and residential development in coastal areas (Wilson et al., 1990), and the possibility that pollutant exposure has compromised oyster defense systems has led to considerable research on various aspects of immunotoxicity in these economically important bivalves.

Numerous studies have demonstrated changes in putative bivalve defense-related activities in response to experimental chemical exposure or associated with life in contaminated habitats (Coles et al., 1994, 1995; Chu et al., 2002). Such activities appear to be stimulated by chemicals in some cases and suppressed in others; thus, demonstration of a clear connection between altered defense capacity and increased disease susceptibility in oysters has been elusive (Oliver and Fisher, 1999). Experimental exposure to tributyltin (TBT) exacerbated *P. marinus* infections and related mortality of *C. virginica* in two recent studies, but evidence of change in defensive functions was lacking (Anderson et al., 1996; Fisher et al., 1999). How to best characterize the bivalve defense process itself remains a challenge. For example, certain cellular functions (such as oxyradical generation) vital to effective immunity in higher animals may not serve any defensive function in oysters (Bramble and Anderson, 1999).

Two recent field surveys of FL oysters found positive relationships between certain trace metals and organic compounds with putative defensive activities, but conclusions were limited in part because analysis of contaminant burden relied on pooled oyster tissues (Fisher et al., 2000; Oliver et al., 2001). To further elucidate relationships between contaminants and defense functions, we employed measurements of contaminant burdens and defense activities of individual oysters that allowed valid statistical comparisons to be made with individually-measured defense activities, as well as application of multivariate analysis to explore relationships between multiple pollutants and multiple defense activities. In addition, evaluation of defense activities employed an integrative assay of hemocyte bactericidal function, rather than relying solely on measuring separate phases of the phagocytic response such as hemocyte locomotion or oxyradical generation.

The study took place in Pensacola Bay, an estuarine ecosystem in the NW panhandle region of Florida. Several small bayous enter the bay and receive diverse point and non-point pollution inputs. Resident oysters were collected from one such site, Bayou Chico (BC), which harbors several marinas and is surrounded by residential and some industrial development. The bayou receives nutrient, trace metal and organic contamination. Oysters were also collected from East Bay (EB), an open water site with no apparent point sources of pollution nearby. However, non-point runoff from relatively sparse coastal residential development is likely as well as cumulative input from upstream industrial activity since the Blackwater River enters EB about 0.8 km from the oyster collection site. The objective of the study was to compare resident eastern oysters from these contrasting sites to determine whether: (1) significant differences exist between the two sites in average oyster defense measurements or tissue chemical accumulation, (2) significant relationships exist between any oyster defense activity and the concentration of a specific chemical in their tissues and (3) significant relationship exists between multiple defense-related activities of oy-

sters and their tissue concentrations of metals, assessed using canonical correlation analysis.

2. Materials and methods

2.1. Oyster collection

Oysters (20 on each date) were collected from BC on August 5 and 7, 1998 by carefully scraping them off hard substrates, and from EB on August 17 and 18, 1998 using hand tongs (Fig. 1). Animals were selected that were at least 8 cm in shell height. Temperature and salinity at time of collection were recorded with a thermometer and refractometer. Animals were returned to the US Environmental Protection Agency's Gulf Ecology Division immediately after collection for processing. Various subsets of oysters were subjected to tests of defense-related activities and chemical analyses (Table 1).

2.2. Defense/physiological assays

All 40 oysters from each site were processed as follows: each oyster was notched at the posterior shell edge with a grinder, the interior mantle cavity was washed using a squirt bottle containing filter-sterilized seawater, and hemolymph (0.8–4.0 ml) was withdrawn from the adductor muscle using a

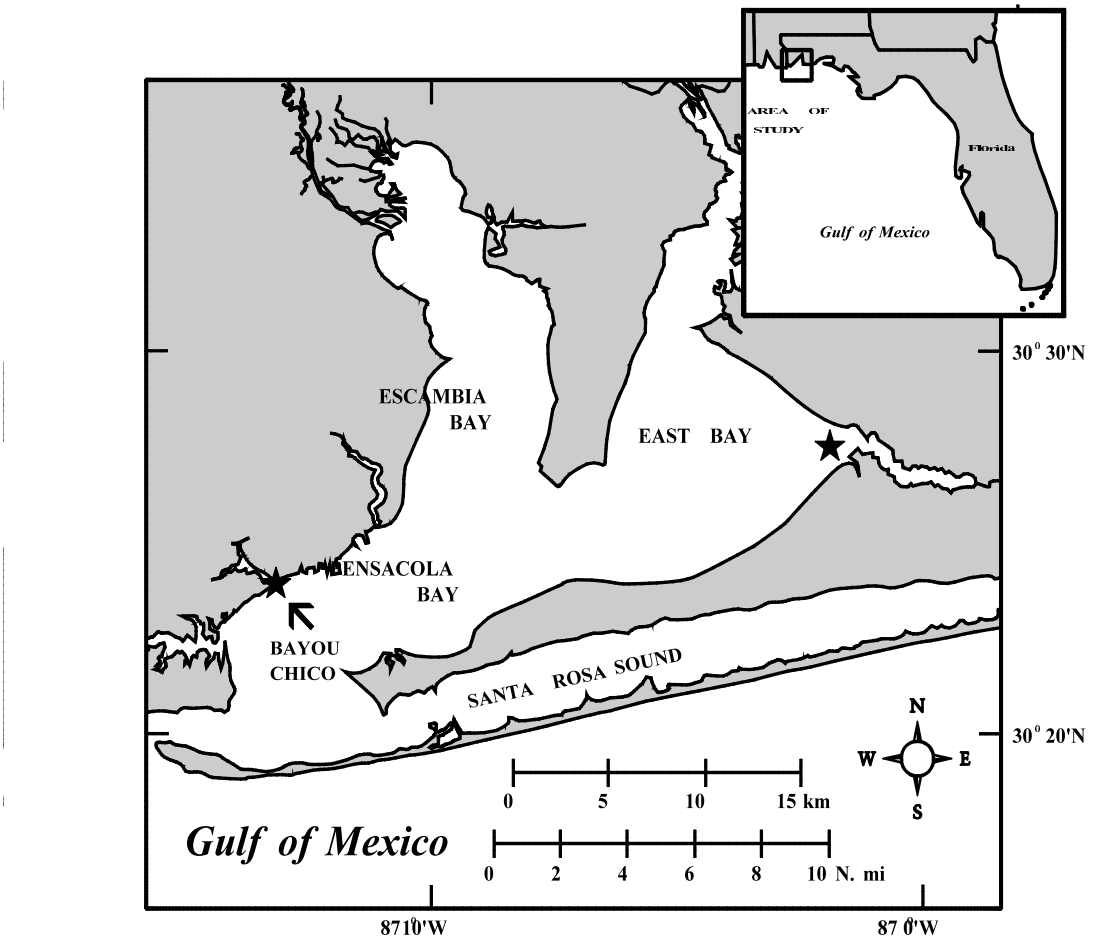

Fig. 1. Map of Pensacola Bay system showing oyster collection sites.

Table 1
Analyses on subsets of oysters collected in August 1998 from BC and EB, Pensacola, FL, and assessed for various defense-related/physiological functions (see text) and tissue chemical accumulation

Site	Collection date	Defense measurements	Trace metals	BTs	PCBs	PAHs
BC	8/5	HC, KI, LYS, PRO (20)	NA	20	NA	NA
BC	8/7	HC, KI, LYS, PRO (20)	20	NA	10	10
BC totals		40	20	20	10	10
EB	8/17	HC, KI, LYS, PRO (20)	NA	20	NA	NA
EB	8/18	HC, KI, LYS, PRO (20)	20	NA	10	10
EB totals		40	20	20	10	10

Numbers in parentheses indicate the number of oysters analyzed. NA, no analysis.

3-ml syringe and 22-gauge needle. The number of hemocytes per milliliter for each oyster was counted at 400X using a hemocytometer.

Hemocyte bacterial killing index (KI) against *Vibrio parahaemolyticus* was measured using the method of Volety et al. (1999). Briefly, this colorimetric technique utilizes tetrazolium dye reduction to indirectly quantify bacterial viability in the presence of hemocytes versus that of hemocyte-free controls. Based on hemocytometer counts, hemocytes were delivered at a constant number for all KI tests (10^5 per well of a microtiter plate), and the bacterial challenge was 10 *V. parahaemolyticus*:1 hemocyte.

After hemocyte enumeration and initiation of the KI assay, hemolymph samples were centrifuged ($2900 \times g$ for 5 min) and supernatant (cell-free hemolymph) was frozen at $-20\,^{\circ}C$. Frozen cell-free hemolymph samples (all 40 from each site) were thawed and analyzed for protein using the Pierce BCA Protein Assay kit as modified by Fisher et al. (1996b). Lysozyme was quantified by measuring the ability of cell-free hemolymph to degrade suspensions of bacteria *Micrococcus lysodeikticus* (Sigma Chemical Co.) as detailed in Fisher et al. (1996a).

2.3. Chemical analysis

Chemical residues in whole oyster tissue were analyzed by Batelle Laboratory, Duxbury, MA[1]. Trace metals were analyzed on 20 oysters from each site and included silver (Ag), aluminum (Al), arsenic (As), barium (Ba), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), manganese (Mn), nickel (Ni), lead (Pb), molybdenum (Sb), selenium (Se), tin (Sn) and zinc (Zn). Tissue digestion procedures followed EPA Method 200.3, modified by substituting aqua regia for the digestion acid (EPA, 1991a). Mercury was analyzed using cold-vapor atomic absorption spectroscopy (Bloom and Crecelius, 1983) and the remaining metals were analyzed by inductively coupled plasma mass spectroscopy following EPA Method 200.8 (EPA, 1991b).

Organic tissue residues were measured on a subset of the same 20 oysters from each site that were analyzed for trace metals. Ten oysters were selected that were of sufficient size to allow both metals and organic analyses to be performed on homogenized tissues. Tissues were extracted following Batelle SOP 5-190, Tissue and Sediment Extraction for Trace Level Semi-volatile Organic Contaminants, and then split into two aliquots for polycyclic aromatic hydrocarbon (PAH) analysis via gas chromatography (GC)/mass spectrometry (MS) (Batelle SOP 5-257, Identification and Quantification of Polynuclear Aromatic Hydrocarbons by Gas Chromatography/Mass Spectrometry), and polychlorinated biphenyl (PCB) analysis via GC with electron capture detection (Batelle SOP 5-128, Identification and Quantification of PCBs (by Congener and Aroclor) and Chlorinates Pesticides by Gas Chromatography/Electron Capture Detection (Modified SW846

[1] Mention of trade names or companies does not imply endorsement by the US Environmental Protection Agency.

Method 8081). Analysis of organic chemicals (PAH, PCB and pesticides) included those analytes listed in Fisher et al., 2000. Total PAH and pesticide concentrations (ng/g dry weight) were calculated by summing the concentrations of detected analytes, and total PCBs (ng/g dry weight) by summing 18 analytes targeted by NOAA's National Status and Trends Program and multiplying this total by 2.19 to estimate total PCBs (Fisher et al., 2000; Oliver et al., 2001).

Tri-, di-, and monobutyltin were measured using GC/FPD in tissue of 20 oysters from each site according to Batelle SOP 5-196-04, Measurement of Butylating Species in Tissues and Sediment/Soil. Concentrations of all butyltins (BTs) were summed to generate total BTs.

2.4. Statistical analysis

Site means for the oyster measurements HC, KI, LYS and PRO ($n = 40$ per site) were compared using Student's t-tests with $P < 0.05$ adopted as the criterion for significance (SAS, Inc., Cary, NC). Appropriate power transformations were applied when necessary to meet the statistical assumptions of normality and homoscedasticity (constant variance) associated with the t-test using Malaeb's Box-Cox power transformation program (Table 2) (Malaeb, 1997).

Student's t-tests were also used to compare site means of chemical analytes in oyster tissue including individual and total trace metals, total PAHs, pesticides and PCBs. Transformations were required for most metal data to stabilize the variance and achieve normality (Table 3). For several analytes, including BTs and PCBs, and most pesticides no detectable concentrations were found in EB oysters so no statistical analysis was required.

Relationships between oyster defense-related measurements and individual and total chemical contaminants were explored using Pearson's product moment correlation with $P < 0.05$ adopted as the criterion for significance. These simple correlations encompass inter-oyster variability in both physiology and chemical content, with $n = 20$ for correlations with organic chemicals and $n = 40$ for metals.

Metal residue data and oyster defense-related measurements collected on 20 oysters per site were combined and analyzed using canonical correlation analysis to ascertain whether there was a significant association between the three defense characteristics and the 16 metals. In the presence of large numbers of pairwise comparisons, such as in our case where there are 48 (3×16) such comparisons to consider, conducting Pearson's correlation analysis may yield contradictory conclusions. Therefore, canonical correlation analysis was applied to identify the association between the combined defense and metal variables such that the correlation between canonical variables (designated U for defense and V for metals) is maximized. Using SAS PROC CANCORR, the optimal correlation was determined between linearly combined sets of variables for defense and tissue metals. Organic contaminant data could not be included in the canonical correlation analysis because the number of oysters from which both defense and organic contaminant data were available was insufficient ($n = 10$ per site) for this test.

Table 2
Average defense-related/physiological characteristics of oysters collected in August 1998 from BC and EB, Pensacola, FL

	BC	EB	P-value	Transformation
HC	**4.51** (2.34)	1.90 (0.86)	0.0001	Natural log
KI	**61.34** (31.10)	2.79 (9.80)	0.0001	None
LYS	**11.19** (5.09)	7.29 (4.94)	0.0008	None
PRO	**4.48** (1.71)	2.75 (1.14)	0.0001	Square root

Standard deviations are given in parentheses, P-values indicate significance of difference between site means, $n = 40$ from each site for all variables, and the highest mean is given in bold for measurements that differed significantly between sites. HC, hemocyte count (10^6 ml^{-1}); KI, bacterial killing index; LYS, serum lysozyme (μg/ml); PRO, serum protein (mg/ml).

Table 3
Average individual and total trace metals (μg/g dry weight), BTs (ng/g dry weight), and total PAHs (ng/g dry weight), PCBs (ng/g dry weight) and pesticides (ng/g dry weight) measured in oyster tissue collected in August 1998 from BC and EB, Pensacola, FL

	BC	EB	*P*-value	Transformation
Al	144.9 (114.5)	**584.8** (406.4)	0.0001	Natural log
Cr	0.746 (0.316)	**1.888** (0.838)	0.0001	Natural log
Mn	**43.06** (24.35)	22.91 (10.37)	0.0014	Natural log
Fe	359.4 (85.1)	**707.0** (273.9)	0.0001	Natural log
Ni	1.89 (0.55)	2.09 (0.35)	0.1200	Natural log
Cu	**1672.4** (796.3)	125.1 (60.0)	0.0001	Natural log
Zn	**13460** (3354)	3670 (1380)	0.0001	Natural log
As	13.26 (1.78)	13.12 (3.19)	0.6446	Natural log
Se	3.67 (0.55)	3.84 (0.64)	0.3654	None
Ag	0.814 (0.368)	**5.472** (1.820)	0.0001	Natural log
Cd	3.51 (0.73)	**6.65** (1.51)	0.0001	Natural log
Sn	**0.291** (0.057)	0.101 (0.056)	0.0001	Natural log
Sb	0.024 (0.0071)	**0.044** (0.0194)	0.0001	Inverse 1/4 root
Ba	1.13 (0.33)	**2.87** (2.37)	0.0001	Inverse square root
Pb	1.04 (0.32)	0.96 (0.35)	0.4517	Natural log
Hg	0.188 (0.035)	**0.416** (0.090)	0.0001	Natural log
Total metals	**15705.8 (3806.2)**	5142.7 (1537.7)	0.0001	None
DBT	**105.39** (22.51)	0		
TBT	**583.03** (744.72)	0		
BTS	**688.42** (755.62)	0		
Total PAHs	**6224.9 (1973.8)**	69.3 (36.9)	0.0001	None
Total PCBs	**368.1 (368.5)**	0		
Total pesticides	**83.8 (81.6)**	6.9 (3.1)	0.0003	Inverse 1/2 root

Standard deviations are given in parentheses, *P*-values indicate significance of difference between site means, the highest mean is given in bold where significant differences were found between sites, $n = 20$ oysters per site for metals and BTs, and $n = 10$ oysters per site for organic chemicals.

3. Results

3.1. Defense/physiological assays

Average hemocyte counts (HCs), hemocyte bacterial killing activity (KI), serum lysozyme (LYS) and serum protein were all significantly higher in oysters from BC compared to those from EB (Table 2).

3.2. Chemical analysis of oyster tissues

All inorganic trace metals targeted in the analysis were detected in oyster tissue from both BC and EB. Of the 16 metals, 12 showed significant differences in average concentrations between BC and EB (Table 3). Oysters from BC had significantly higher concentrations of Mn, Cu, Zn and Sn, while EB oysters had significantly higher Al, Cr, Fe, Ag, Cd, Sb, Ba and Hg. Total metal content was significantly greater in oysters from BC and no BTs were detected in EB oysters (Table 3).

Of 24 PAH analytes measured, all were detected in oyster tissue from BC while only six were detected in oysters from EB. Total PAH concentration in BC oysters was significantly higher than that in EB oysters (Table 3) and in no case was the average concentration of a given PAH in EB oysters higher than that found in BC oysters. Of 43 PCB/pesticide analytes measured, 27 were detected in BC oysters while only two pesticides and no PCBs were detected in oysters from EB. The total pesticide concentration in BC oysters

was significantly higher than that in EB oysters (Table 3), despite one exception of oxychlor, which was higher in oysters from EB.

3.3. Simple correlation analysis

Using data from both sites, Pearson's product moment analysis found oyster HCs to be positively and significantly correlated with Cu, Zn, Sn, DBT, total metals (Table 4), 20 of 24 individually measured as well as total PAHs (Table 5), and with the three of 43 PCBs (r-values ranged from 0.468 to 0.513). Significant negative correlations were found between HC and Al, Cr, Ag, Cd, Ba, and Hg (Table 4).

Oyster hemocyte KI was positively and significantly correlated with Mn, Cu, Zn, Sn, DBT, total metals (Table 4), 23 of 24 individual PAHs, total PAHs, total PCBs (Table 5) and 21 of 43 individual PCB/pesticide analytes (data not shown). r-values for significant correlations between KI and PCB analytes were all positive, with the exception of one negative correlation with oxychlor, and ranged from 0.457 to 0.847. Hemocyte KI was negatively correlated with Al, Cr, Fe, Ag, Cd, Sb, Ba and Hg (Table 4).

LYS concentration was positively correlated with DBT and negatively correlated with Ag and Cd (Table 4). There were no significant associations between lysozyme and any PAHs, PCBs or total contaminants. Oyster serum protein level was positively correlated with Zn, Sn, Pb, DBT, total metals (Table 4), seven of 24 PAHs (Table 5) and PCB C1444 ($r = 0.4668$, $P < 0.0380$). Protein was correlated negatively with Ag, Cd, Sb, Ba and Hg (Table 4).

3.4. Canonical correlation

Canonical correlation analysis detected a significant positive relationship between oyster defense measurements and tissue metal concentrations. Results of the initial analysis including all variables indicated that Al, Zn, Se, As, Cr, Mn and Ba be removed from the set of metal variables because their relative contribution to the association between the two sets was minimal. In the final analysis, we used all three defense variables, HC, KI and LYS, and nine

Table 4
Pearson's correlation coefficients from simple correlation analysis (sites combined) between defense/physiological measurements and tissue metal concentrations in individual oysters

	KI	HC	LYS	PRO
Ag	−0.6889**	−0.4373**	−0.3260*	−0.4903**
Al	−0.4871**	NS	NS	NS
Ba	−0.3865*	−0.3229*	NS	−0.3314*
Cd	−0.6183**	−0.3814*	−0.3593*	−0.5664**
Cr	−0.5714**	−0.3196*	NS	NS
Cu	**0.6722****	**0.4619****	NS	NS
Fe	−0.5429**	NS	NS	NS
Hg	−0.7018**	−0.4407**	NS	−0.4892**
Mn	**0.3635***	NS	NS	NS
Pb	NS	NS	NS	**0.3198***
Sb	−0.4680**	NS	NS	−0.3409*
Sn	**0.7020****	**0.4846****	NS	**0.5260****
Zn	**0.7384****	**0.4883****	NS	**0.3841***
TBT	NS	NS	NS	NS
DBT	**0.6824****	**0.6142****	**0.4973****	**0.4973****
Total (inorganic) metals	**0.7312****	**0.4902****	NS	**0.3779***

$n = 40$ for individual and total metals, 20 oysters per site. NS, not significant. Positive correlation coefficients are shown in bold type.
* Significant at $P < 0.05$.
** Significant at $P < 0.01$.

metal variables, Fe, Cu, Ag, Cd, Sb, Sn, Ni, Pb and Hg. The canonical variables U_1 (defense variables $= 0.240 \times \text{HC} + 0.793 \times \text{KI} + 0.363 \times \text{LYS}$) and V_1 (metal variables $= 0.262 \times \text{Fe} + 0.153 \times \text{Cu} - 0.284 \times \text{Ag} + 0.153 \times \text{Cd} - 0.111 \times \text{Sb} + 0.667 \times \text{Sn} + 0.120 \times \text{Ni} - 0.235 \times \text{Pb} - 0.305 \times \text{Hg}$) showed a high positive canonical correlation of magnitude $r = 0.864$, $P < 0.0019$.

Table 5
Pearson's correlation coefficients from simple correlation analysis (sites combined) between defense/physiological measurements and individual PAHs, and between defense/physiological measurements, and total PAHs and PCBs in oysters from two sites in Pensacola, FL, $n = 20$ for individual and total organic chemicals, 10 oysters per site

	KI	HC	PRO
Naphthalene	NS	NS	0.6819**
2-Methylnaphthalene	0.8728**	0.4939*	0.5103*
1-Methylnaphthalene	0.7525**	NS	NS
Biphenyl	0.6494**	NS	NS
2,6-Dimethylnaphthalene	0.8400**	0.4796*	NS
2,3,5-Trimethylnaphthalene	0.8271**	0.5011*	NS
Acenaphthylene	0.6966**	NS	NS
Acenaphthene	0.8303**	0.5098*	NS
Fluorene	0.8188**	0.4907*	NS
Phenanthrene	0.8296**	0.4794*	NS
Anthracene	0.8014**	0.4873*	NS
1-Methylphenanthrene	0.8174**	0.4576*	NS
Fluoranthene	0.8244**	0.4692*	NS
Pyrene	0.8304**	0.4657*	NS
Benz[*a*]anthracene	0.8326**	0.4874*	NS
Chrysene	0.8279**	0.4711*	NS
Benzo[*b*]fluoranthene	0.8121**	0.4724*	NS
Benzo[*k*]fluoranthene	0.7359**	0.4560*	NS
Benzo[*e*]pyrene	0.8499**	0.4767*	NS
Benzo[*a*]pyrene	0.7031**	0.4805*	0.4984*
Perylene	0.8011**	0.5233*	0.4726*
Indeno[1,2,3-*c*,*d*]pyrene	0.6857**	0.4544*	0.5227*
Dibenz[*a*,*h*]anthracene	0.6860**	0.4953*	0.4659*
Benzo[*g*,*h*,*i*]perylene	0.7900**	0.4686*	0.5313*
Total PAH	0.8403**	0.4859*	NS
Total PCB	0.6465**	NS	NS

NS, not significant. No significant correlations were found between LYS and any individually measured or summed organic contaminants.

* Significant at $P < 0.05$.

** Significant at $P < 0.01$.

4. Discussion

In previous field surveys of resident oysters in five Florida estuaries, defense-related responses, including hemocyte number, locomotion and some measures of oxyradical production, were elevated in oysters from polluted habitats (Fisher et al., 2000; Oliver et al., 2001). The current intensive study of oysters from two sites in Pensacola Bay corroborates these findings in several ways: (1) higher HC, KI and LYS were found at BC, the site with the highest average total contaminants; (2) simple correlation analysis showed positive relationships between defense activities and certain metals, including Cu, Sn and Zn, and many organic contaminants; and (3) canonical correlation analysis revealed a significant positive relationship between defense variables and tissue levels of trace metals. Assessment of individual variability in both defense and chemical content allows greater statistical certainty in these conclusions, as contrasted with the previous field surveys which relied upon pooled samples for tissue chemical analyses.

Concentrations of total metals, PAHs, and pesticides in BC oysters significantly exceeded those in EB oysters by approximate factors of $3\times$, $90\times$ and $12\times$. No BTs or PCBs were detectable in EB oysters. Despite finding some trace metals (such as Al and Ag) significantly higher in EB oysters, BC was clearly the more contaminated site. All physiological measures were higher in BC oysters, with KI exhibiting the most dramatic difference between sites (Table 2). A highly significant and positive canonical correlation between defense measurements and metal contaminants supports earlier findings of heightened defense activities in oysters exposed in nature to chemical contaminants. It remains possible that one or more of the significantly higher metal contaminants in oysters at EB suppressed defense activities. But, with the possible exception of Ag and Al, these significant differences were only marginally higher and the magnitude of contamination for these elements was minor compared to BC contamination from Cu, BTs, PAHs and PCBs. Despite repeated observation of positive simple correlations be-

tween defense activities and specific metals such as Cu, Sn and Zn, we note that there were also several significant negative correlations with other metals such as Ag, Cd, Cr and Hg (Table 4). Hence, we rely on the more inclusive canonical procedure, indicating that the defense measurements employed were, overall, positively correlated with a subset of metals.

Canonical analysis was not applied to organic contaminant data, yet all significant simple correlations between defense measurements and organic contaminants were positive. In the case of correlations with KI, high coefficients were driven by the preponderance of zero values for organic analytes and KI in oysters from EB. Given that total PAH and PCB/pesticide content of BC oysters exceeded that by EB oysters by such large factors, organic contamination may be a factor in the observed differences in defense responses between the two sites.

Compared to the two previous FL field studies, BC oysters had high metal content, but total PAHs were 20-fold below that found in the most contaminated site in St. Andrew Bay in 1995 (Oliver et al., 2001), and total PCBs were less than half that of the "worst" Tampa (Fisher et al., 2000) and St. Andrew Bay sites. Although samples were disparately composed of pooled tissue versus individual tissues in the current report, gross comparisons reinforce the hypothesis that increased HC may be associated with exposure to metals. For example, the most severely organically contaminated site in St. Andrew Bay in 1995 contained high PAHs and PCBs (159,366 and 1929 ng/g dry weight, respectively), but moderate (4610 μg/g) total metals and an average HC of 2.45×10^6 cells/ml hemolymph. By contrast, one 1993 Tampa Bay site had low-moderate PAHs and PCBs (11,026 and 624 ng/g dry weight, respectively) but high (11,886 μg/g) total metals and average HC of 4.92×10^6 cells/ml hemolymph (Fisher et al., 2000; Oliver et al., 2001). The contaminant profile in BC oysters resembles this Tampa Bay site, with low-moderate total PAH and PCBs (6225 and 368 ng/g dry weight, respectively), high metals (15,706 μg/g) and high average HC of 4.51×10^6 cells/ml hemolymph. Oysters from EB were very low in organic contaminants, moderate in total metals (5143 μg/g) and had low average HC of 1.90×10^6 cells/ml hemolymph.

By applying the KI method as a defense endpoint, our results demonstrate not only greater hemocyte numbers in contaminated oysters but a heightened bactericidal capacity, at least in vitro, by hemocytes from contaminated sites. Coupled with dramatically higher numbers, these defensively active cells may provide an elevated level of defense against in vivo bacterial challenge as well. Potential interactive factors include disease status, as oysters with moderate to heavy infestations of *P. marinus* and certain metazoan parasites respond with elevated numbers of circulating hemocytes and oxyradical production (Anderson et al., 1995; Fisher et al., 2000). Moreover, since *P. marinus* infections may be exacerbated by exposure to both metal and organic contaminants (Chu and Hale, 1994; Anderson et al., 1996; Fisher et al., 1999; Chu et al., 2002), attributing changes in defense directly to contaminant exposure is elusive in field experiments. Since it was necessary to sacrifice the whole oyster to provide requisite tissue for contaminant analysis, we did not assess parasite burdens. Fisher et al. (2003) reported no significant difference between *P. marinus* levels in oysters from BC and a reference site in Pensacola Bay during September 1998, nor any metazoan parasitic infestations which might cause elevated defense responses. We also note that salinity and temperature, two primary environmental drivers of *P. marinus* infection intensity, were similar at EB and BC at the time oysters were collected.

LYS, shown to be produced by hemocytes and released into the serum during phagocytosis (Cheng et al., 1975), was higher in BC oysters. This could reflect lysosomal destabilization associated with uptake of pollutants, a phenomenon reported for *Mytilus edulis* exposed to inorganic (Pickwell and Steinert, 1984) and organic contaminants (Moore et al., 1978). Since lysosomes appear to serve a storage role for both inorganic and organic pollutants (Stauber, 1950; Ruddell and Rains, 1975; Pirie et al., 1984), incorporation of these chemicals may reduce integrity of lysosomal and cellular membranes, resulting in "leakage" into the serum. However, even if lysozyme production and release by BC oyster hemocytes

was equal to that of EB oyster hemocytes, the increased number of circulating hemocytes in BC oysters could account for the increased LYS concentrations. Total serum protein was also higher in BC oysters and may indicate some difference in nutrient transport or storage patterns between these oyster populations related to the gametogenic cycle (Fisher and Newell, 1986; Akashige, 1990; Fisher et al., 1996a).

Numerous observations of heightened oyster defense activities in chemically stressed oysters exist, as do examples of possible immunosuppression. Consistent with our results include reports of increased hemocyte numbers in mussels experimentally exposed to Cd (Pipe and Coles, 1995; Coles et al., 1995), Cu (Pickwell and Steinert, 1984) and fluoranthene (Coles et al., 1994), as well as the FL field surveys of *C. virginica* already noted. In another experiment, deployment of common stock oysters at BC for 3 months resulted in greater hemocyte KI compared to oysters held at a reference site (Fisher et al., 2003). These studies were interpreted in the context of the bivalves' capacity to mount an effective internal defense, but elevated HC may also reflect the animal's detoxification processes in that hemocytes may respond to sequester and/or eliminate contaminants (Ruddell and Rains, 1975; Pirie et al., 1984). Similarly, increased KI against *V. parahaemolyticus* may be a concurrent byproduct of increased hemocyte capacity to pinocytose these elements.

Concurrent assessment of pollutants and putative defense functions of oysters at two sites supports previous connections between high tissue contaminant levels and elevated defense parameters. Despite these connections and the positive canonical correlation between grouped defense and metal variables, identifying specific contaminants responsible for the changes in oyster physiology will require controlled laboratory exposures. Challenges faced when evaluating potential effects of pollutants on shellfish defenses include high individual variability in responses of interest and chemical accumulation, a nebulous understanding of the most relevant components of bivalve immunology on which to focus such efforts, and the immense plasticity and resiliency of bivalve physiological processes in response to parasites, diseases and other environmental factors (Oliver and Fisher, 1999). Despite the complexity of the problem, devastating disease-induced losses to the oyster industry and damage to ecosystems reliant on oyster populations as a critical trophic component demand that potential synergism between environmental pollution and disease/defense processes continue to be rigorously examined.

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